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The *Salmonella* Pathogenicity Island 2-Encoded Type III Secretion System Is Essential for the Survival of *Salmonella enterica* Serovar Typhimurium in Free-Living Amoebae[▽]

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Free-living amoebae represent a potential reservoir and predator of *Salmonella enterica*. Through the use of type III secretion system (T3SS) mutants and analysis of transcription of selected T3SS genes, we demonstrated that the *Salmonella* pathogenicity island 2 is highly induced during *S. enterica* serovar Typhimurium infection of *Acanthamoeba polyphaga* and is essential for survival within amoebae.

The importance of free-living protozoa, such as amoebae, as environmental reservoirs of food-borne pathogens is becoming increasingly recognized (1, 6, 9, 18). Such interactions may also have highly significant physiological implications, as amoebic passage of *Legionella pneumophila* enhanced bacterial virulence (3) and could resuscitate viable but nonculturable cells (20). *Salmonella enterica* serovars Typhimurium and Dublin have been shown to survive within *Acanthamoeba polyphaga* and *Acanthamoeba rhysodes* (5, 22), and induction of *fis* transcription, indicative of bacterial proliferation within contractile vacuoles, has been reported (3). The role of the *Salmonella* pathogenicity island 1 (SPI-1)-encoded type III secretion system (T3SS-1), which mediates forced bacterial uptake via subversion of actin dynamics, is unclear; however, an *S. enterica* serovar Dublin Δ *hilA* mutant lacking a key transcriptional activator of SPI-1 entered and survived within *A. rhysodes* at a level similar to that of the parent strain (22). A second type III secretion system encoded by SPI-2 (T3SS-2) and the PhoPQ two-component regulatory system are known to play key roles in the intracellular survival of *Salmonella* in mammalian cells, but their roles in interactions with protozoa have not been reported. In this study we investigated the roles of T3SS-1, T3SS-2, and PhoP in entry and survival of *S. enterica* serovar Typhimurium in *A. polyphaga* using defined mutant strains. Additionally, we quantified transcription of the *S. enterica* serovar Typhimurium SPI-1 gene *sipC* and the SPI-2 gene *sseC* (which encode components of the type III secretion translocator) within *A. polyphaga*.

A gentamicin protection assay modified from that used to quantify *Salmonella* invasion of avian cells was used to assess uptake and intracellular survival in *Acanthamoeba polyphaga* Linc-1 grown in axenic culture in peptone-yeast extract-glucose medium (PYG) (1, 2). Amoebae were grown to 5×10^6 cells per ml initially in flasks and then in 24-well plates. *S. enterica*

serovar Typhimurium strain F98 and previously described variants with mutations in the SPI-1 gene *spaS* and in the SPI-2 gene *ssaU* (genes well characterized for changes in their phenotype for virulence, cell invasion, and survival within macrophages [10, 11, 23]) and in *phoP* (13) were grown at 37°C in Luria-Bertani (LB) broth with shaking at 150 rpm to late logarithmic phase and added to amoebae at a multiplicity of infection (MOI) of 10. After 1 h of incubation at 37°C, the plates were centrifuged at $50 \times g$ for 5 min. The medium was removed from the plates and replaced with PYG containing gentamicin sulfate at $100 \mu\text{g ml}^{-1}$. The plates were incubated at 37°C for 30 min and then centrifuged again before the medium was replaced with PYG containing $20 \mu\text{g ml}^{-1}$ gentamicin to inhibit growth of any *Salmonella* released from lysed amoebae. After 1, 4, 24, or 48 h of incubation, the medium was removed and amoebae were lysed by the addition of distilled water, and then serial dilutions of the lysates were plated onto nutrient agar to enumerate *Salmonella* bacteria after 24 h of incubation at 37°C. Following challenge, between 10^3 and 10^4 CFU of *S. enterica* serovar Typhimurium F98 per ml of cultured amoebae were recovered from amoebic cells (Fig. 1). Both the parent and Δ *spaS* mutant were recovered at similar levels from 1 h postinfection to 48 h postinfection. In contrast, recoveries of the Δ *ssaU* mutant and Δ *phoP* mutant were significantly lower than recovery of the parent strain ($P < 0.01$) at each time point. There was also a decline in the number of viable bacteria recovered over time. The viability of amoebic cultures was slightly reduced following *Salmonella* infection, which may contribute to the gradual decline in bacterial numbers recovered over time. Counts of viable amoebae by microscopy and measurement of cytotoxicity by use of a commercial assay (Cytotox96; Promega, Southampton, Hampshire, United Kingdom) showed between 47 and 57% cell death in *Salmonella*-infected cells at 48 h postinfection, whereas uninfected controls showed 36% cell death. Differences in amoebic death between mutant strains were minimal and would not account for the differences in bacterial survival.

To determine intra-amoebic transcription of the T3SS genes *sseC* and *sipC* and the housekeeping gene *yejA* as an internal standard, invasion assays were performed in 20-ml flasks as

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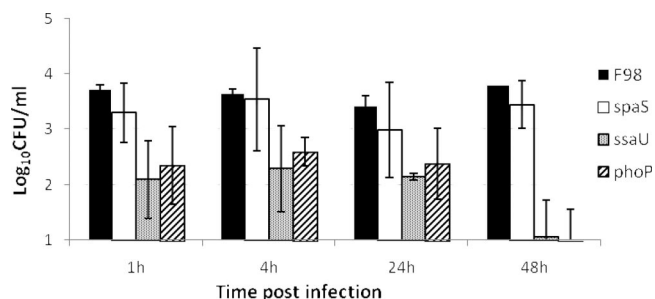


FIG. 1. Survival of *S. enterica* serovar Typhimurium F98 parent and SPI-1 (*spaS*), SPI-2 (*ssaU*), and *phoP* mutant strains within *A. polyphaga* in axenic culture. The values are the mean numbers of *Salmonella* recovered \pm standard errors of the means (error bars) from six replicate experiments. *Salmonella* was added at an MOI of 10 in these experiments.

described above. The *sseC* and *sipC* genes were selected as representatives of the SPI-2 and SPI-1 T3SS, respectively, as the gene products are expressed within the host cell as part of the translocon stabilizing the needle-like apparatus and we have previously demonstrated their expression within porcine and murine macrophages (14). After 1 or 4 h of incubation, flasks were centrifuged to obtain a cell pellet from which total RNA was extracted with TRI reagent and treated in solution with Turbo DNase (Ambion, Inc., Austin, TX), followed by on-column DNase treatment with RNase-free DNase. Transcription at time zero was determined in *Salmonella* grown in LB broth as described above. Real-time reverse transcriptase PCR (RT-PCR) was performed with the MJ Research/Bio-Rad Opticon 2 system with quantitative RT-PCR Mastermix (Eurogentec, Seraing, Belgium) using previously described conditions (14). To quantify transcription, the $2^{-\Delta\Delta Ct}$ method was used for data analysis (12), and transcription was reported as *n*-fold induction normalized to the internal standard and relative to the control at time zero (14). RT-PCR data were analyzed by Student's *t* test with a *P* value of >0.05 considered statistically significant.

Significant induction of both the SPI-1 gene *sipC* and the SPI-2 gene *sseC* was found to occur relative to *yejA* within amoebic cells at 1 h postinfection (Fig. 2), with an approximately sixfold increase over *Salmonella* grown in LB broth ($P > 0.02$). At 4 h postinfection, there was no significant change in transcription of *sipC*. In contrast, a dramatic increase in transcription of the SPI-2 gene *sseC* was found at 4 h. Transcription within amoebic cells was increased over *Salmonella* grown in LB broth by a mean value of 147-fold ($P = 0.001$). Previously we have shown *sseC* to be expressed by *S. enterica* serovar Typhimurium within porcine and murine macrophages at 4 h postinfection (14). A range of SPI-2 genes are also strongly induced following *S. enterica* serovar Typhimurium infection of J774A.1 murine macrophage-like cells (4) and epithelial cells (8).

Taken together, these data indicate that the SPI-2-encoded T3SS influences survival within amoebic cells, as is the case with macrophages. Previously we have shown that mutation of *ssaU* reduces the ability of a range of *Salmonella* serovars to survive within phagocytes and cause systemic infection (10, 11, 23). The failure of the $\Delta phoP$ mutant to survive in *A. polyphaga*

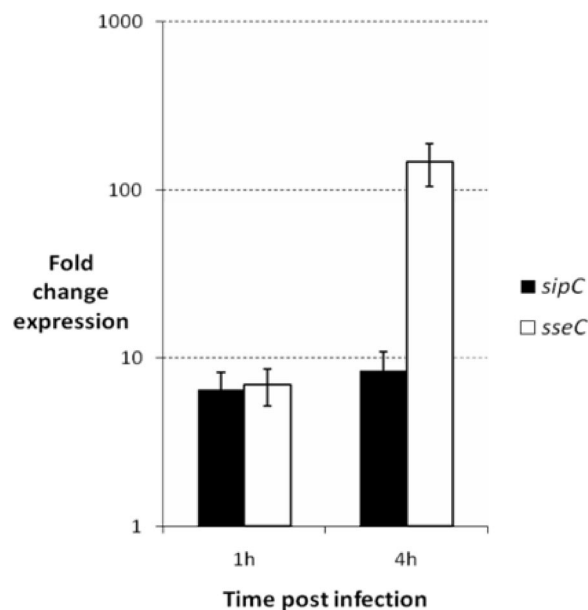


FIG. 2. Relative transcription of SPI-1 (*sipC*) and SPI-2 (*sseC*) genes of *S. enterica* serovar Typhimurium F98 within amoebic cells 1 and 4 h postinfection. *Salmonella* was added to the amoebae at an MOI of 10. Changes in expression were determined by real-time quantitative RT-PCR compared to transcription of bacteria grown in LB broth and using the housekeeping gene *yejA* as an internal standard. Data shown are the mean values for the change in expression \pm standard errors of the means (error bars) from eight replicate experiments.

cells further supports the theory that survival within amoebae and survival within macrophages are largely analogous, as the PhoPQ two-component regulatory system, although associated with regulation of many genes, is key to activation of the SPI-2-encoded T3SS within phagocytes (7, 15). In contrast, mutation of *spaS* had a minor effect on *Salmonella* survival within amoebae, consistent with findings using a $\Delta hilA$ mutant (17). Nevertheless, transcription of the SPI-1 gene *sipC* was induced within amoebae, in contrast to findings with *S. enterica* serovar Typhimurium in J774A.1 murine macrophage-like cells, where *sipC* transcription was downregulated 50-fold compared to growth in culture medium (4). Recently, however, SPI-1 genes were found to be induced after infection of epithelial cells, indicating that the intracellular program of bacterial gene expression is sensitive to cell type (8).

Survival within protozoa may represent an important environmental reservoir of *Salmonella* and confer resistance to predation in the gastrointestinal tracts of ruminants. In addition, such interactions may have exerted an evolutionary pressure leading to bacterial divergence, including variation in the lipopolysaccharide O-side chain of *Salmonella* and the development of virulence factors (16, 24, 25). Recent studies have indicated that Shiga toxin (Stx) of the food-borne pathogen enterohemorrhagic *Escherichia coli* (EHEC) aids resistance to grazing protozoa and may account for the very high *stx* carriage rates in EHEC isolated from ruminants (19). Furthermore, the type II and IV protein secretion systems of *L. pneumophila* aid survival in both waterborne amoebae and alveolar macrophages during human infection (17, 21). Taken together with

our finding that *S. enterica* serovar Typhimurium SPI-2 is induced and required for survival in *A. polyphaga*, such data suggest that traits we primarily consider virulence factors for bacterial pathogenesis in animals and humans may have originally evolved to play other roles in microbial ecology.

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